SOME EFFECTS OF ELECTROLYTES ON COLLAGEN IN SOLUTION*

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SUMMARY

Since collagen forms an aqueous solution only when electrolytes are present, a study of the effects of electrolytes on dissolved collagen was carried out to obtain data which might lead to improved solubilization and fractionation techniques. Salting-out curves of collagen in phosphate buffer solutions were prepared. Dissolved collagen was reconstituted from phosphate buffer and citrate buffer solutions by various methods and the precipitated fibrils showed a variety of structural forms. Fibril morphology was related to solution history and an attempt was made to show that electrolytic environment influenced the aggregation of collagen molecules into organized structures through ion binding and electrostatic interaction.

INTRODUCTION

Extensive research on the composition of animal hide that has been carried out in the past has included X-ray diffraction and amino acid sequence studies which have indicated the helical structure of the collagen molecule and also that these molecules are aggregated in a very orderly fashion to form characteristic fibrils which, in turn, are the principal structural components of all fibrous tissue[1–3]. Considerable work on collagen solutions has also been done by investigators who solubilized collagen through placing fibrous tissue such as hide, tendon and fish swim bladder in contact with aqueous inorganic buffer solutions, and subsequently established the validity of these solutions by precipitating the collagen and showing its similarity to native collagen via electron microscopy[4] or X-ray diffraction. (CROSBY[5], DELAUNAY[6] and HIGHBERGER[7] have prepared comprehensive reviews on this aspect of the subject.) Solution studies require a collagen solution that is monodisperse and of a concentration sufficiently high to yield meaningful data in investigations of its physical properties. This kind of a solution is difficult to achieve because of the low solubility of collagen, the fact that in its native state it is in intimate contact with many other proteins, and the ease with which the helical structure of collagen breaks down to form gelatin[8]. Since collagen forms an aqueous solution only when electrolytes are present, the study that is here being reported was carried out in hope that a better understanding of the

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relation between collagen and inorganic ions in solution would lead to improved solubilizing and fractionating techniques.

In this work the solubility and salting-out of collagen in phosphate buffer, and the effects of electrolytic environment on the morphology of collagen fibers reconstituted from phosphate buffer and citrate buffer solutions were investigated.

EXPERIMENTAL

Natural sources of collagen

Skins of bovine embryos which had been under gestation for periods of five to eight months served as the natural source of collagen for the salting-out experiments. The embryos were obtained from the parent cows at slaughter and were immediately flayed. For the study of the effects of electrolytic environment on the morphology of reconstituted collagen fibers the skins of calves whose ages ranged from three to fifteen months were obtained immediately after the slaughter of the animal. All skins were transported to the laboratory packed in ice. The following day the skins were shaved with an electric clipper (except, of course, in the case of the younger embryos), as much of the fat was cut away as was possible, and the skin was cut into approximately 0.5-in squares with scissors. The skins were kept under ice water except while being clipped and diced.

Solubilization

Solubilization was carried out by keeping a quantity of the diced skin in contact with approximately twice its volume of buffer in a stoppered Erlenmeyer flask for 5 days. This operation and all subsequent ones involving the collagen solutions were carried out in a cold room at a temperature of 6–8°. Three or four times in each 24 h each flask was gently shaken for a few seconds. Finally, the solutions were decanted, filtered through a plug of cotton and centrifuged for 1 h at 3000 $\times$ g to remove suspended debris.

Assay

The collagen concentrations of the solutions were determined by direct weighing. The collagen was reconstituted by dialyzing a definite volume of solution to exhaustion of the electrolyte, washing the precipitated collagen with several changes of acetone and collecting it on a coarse sintered glass filter. After drying overnight exposed to the atmosphere at room temperature the reconstituted collagen was weighed.

Dialysis

Dialysis in all cases was carried out using sacs of regenerated cellulose. The dialysis medium was continually stirred and was changed twice in each 24-h period for a total time of 3 days.

Electron microscopy

Preparations for the electron micrographs were made by air-drying aqueous suspensions of reconstituted collagen on collodion substrates. The preparations were shadowed with Au–Pd (60:40, w/w), and the electron microscope was set to give a magnification of 11000.

Salting-out data

Salting-out curves were prepared using phosphate buffer at pH 8.4 and 0.33 ionic strength as the collagen solvent. The solution of collagen was divided into several aliquot portions, and each aliquot was subjected to the fractionating scheme shown in Fig. 1. Each aliquot was dialyzed against a portion of phosphate buffer of the same

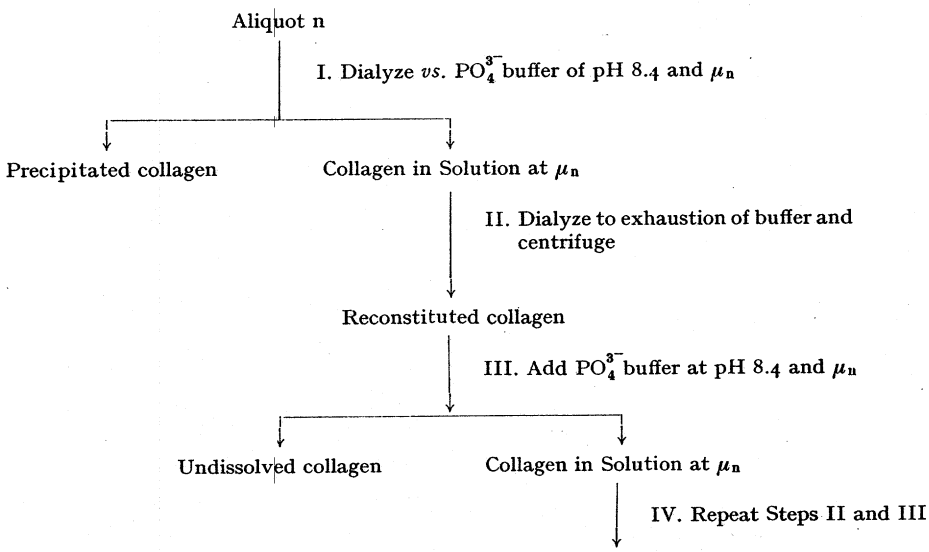


Fig. 1. Flow diagram of collagen fractionation.

pH as the original buffer, but each portion of buffer was of a different ionic strength (Step I). A portion of the collagen in each solution precipitated and this was subsequently removed by centrifugation. Each portion of buffer now held an amount of collagen that corresponded to saturation at that ionic strength. Next, the collagen of each portion of solution was precipitated by dialysis to exhaustion of the inorganic electrolyte, removed by centrifugation (Step II) and then redissolved in buffer identical with that used in the initial dialysis (Step III). This precipitation and redissolving cycle was repeated several times, and each time there was a residue of collagen that would not return to solution.

RESULTS

Salting-out data

The solubility data that resulted after each aliquot was adjusted to its respective ionic strength for the first time at Step I on the flow diagram (Fig. 1) are shown in Fig. 2, where the concentration of collagen remaining in solution is plotted against ionic strength. The curve has a maximum of 0.58 % collagen at approx. 0.33 ionic strength. The progressive decrease in solubility with repeated precipitation and dissolving is illustrated in Fig. 3, where the concentration of collagen in solution at several values of ionic strength is plotted against the number of times the precipitating and dissolving cycle was carried out. The slopes of these curves increase with

increasing ionic strength. In every case the collagen which remained undissolved presented the same appearance when viewed in the electron microscope as that collagen which did dissolve and was subsequently reconstituted.

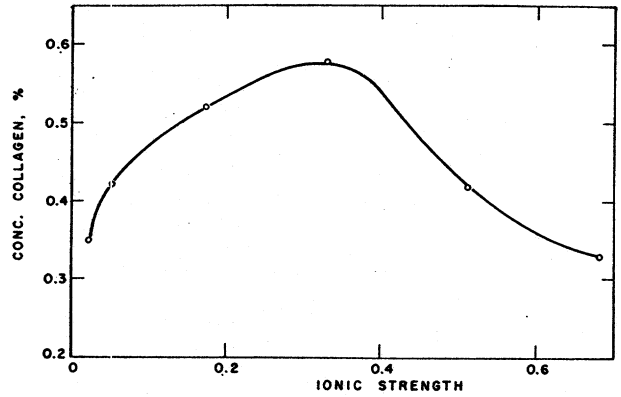


Fig. 2. Collagen salting-out curve. PO_4^{3-} buffer at pH 8.4.

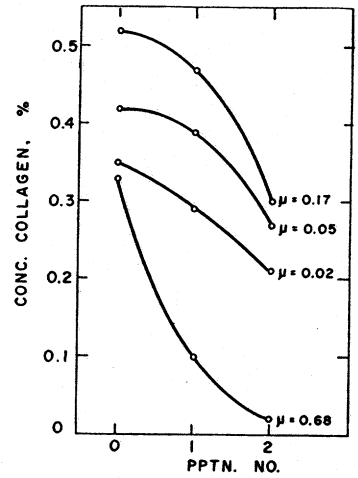


Fig. 3. Effect of repeated salting-out on collagen solubility. PO_4^{3-} buffer at pH 8.4.

Solution history and fibril structure

The effects of dissolving and precipitating collagen using various inorganic salts as dispersing agents were followed by electron microscopy of the reconstituted fibrils.

For the first phase of this study a solution of collagen was prepared using phosphate buffer (pH 8.4) at 0.33 ionic strength as the solvent. As solution of the collagen progressed the pH dropped to 7.6. This, of course, indicates a loss of electrolyte from the liquid phase of the system and can be due to adsorption of buffer ions on the hide, Donnan effect, and binding of ions to collagen molecules in solution.

The collagen of a portion of this solution was reconstituted by dialysis to ex-

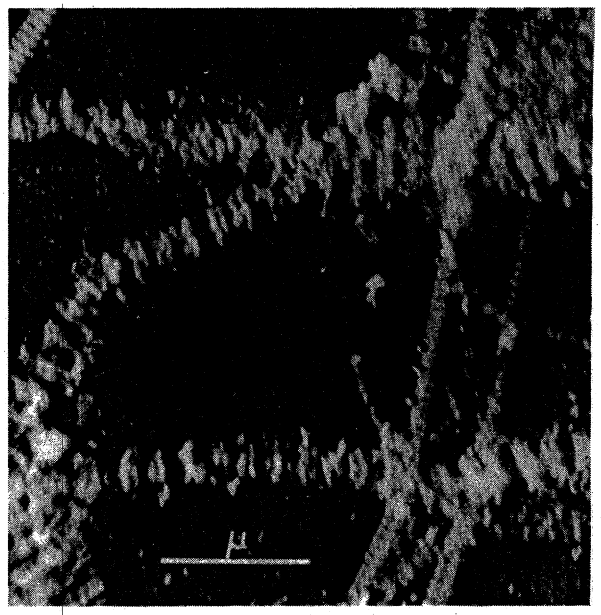


Fig. 4. Collagen fibrils precipitated from phosphate buffer (pH 8.4) at 0.33 ionic strength by dialysis to exhaustion of the inorganic electrolyte.

Fig. 5. Collagen fibrils precipitated from solution in phosphate buffer (pH 8.4) at 0.33 ionic strength by dialysis to exhaustion of the inorganic electrolyte; redissolved in identical phosphate buffer and reprecipitated for a total of three cycles.

haustion of the inorganic electrolyte. An electron micrograph of the resulting fibrils is shown in Fig. 4. Two types of fibrils are present: one of uniform width with regular 640-Å spacing and the other of varying width with an additional superimposed period corresponding to four 640-Å divisions, thus creating a segmented structure. These segmented fibrils bear a very close resemblance to the ones reported by HIGHBERGER, GROSS AND SCHMITT and designated as fibrous long-spacing[9]. These investigators obtained them from solutions of collagen in the acid pH range by adding small amounts of plasma-α-1-acid glycoprotein and then dialyzing against water. GROSS,

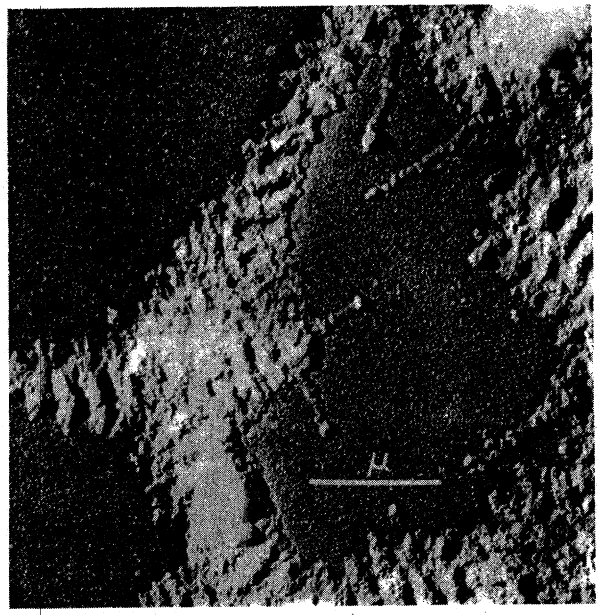
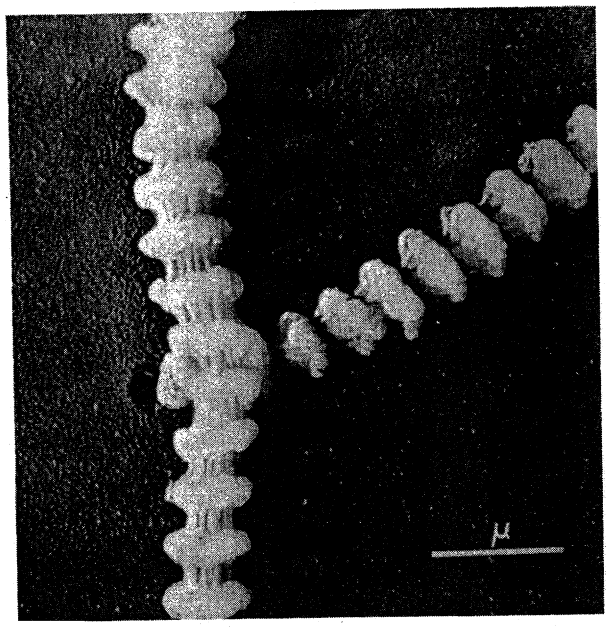


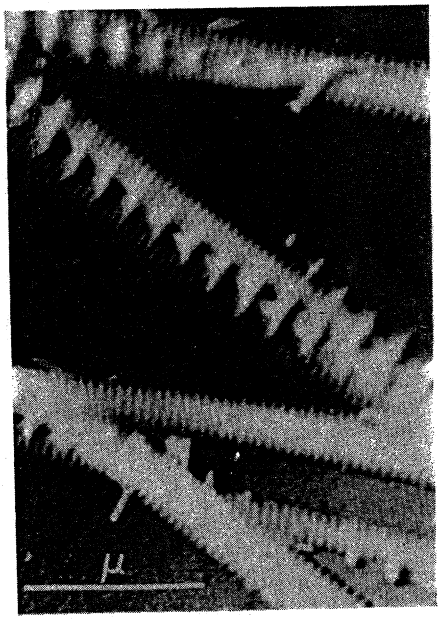
Fig. 6. Collagen fibrils precipitated from solution in phosphate buffer (pH 8.4) at 0.33 ionic strength by salting-out with ammonium sulfate.

HIGHBERGER AND SCHMITT also reported that various mucopolysaccharides as well as mucoproteins could induce the formation of fibrous long-spacing fibrils[10]. When HIGHBERGER, GROSS AND SCHMITT used phosphate buffer as a solvent for collagen and dialyzed the solution against water only amorphous precipitates resulted[9]. In the work here reported, under the experimental conditions that prevailed and with the natural source of collagen that was employed, solutions of collagen in phosphate buffer yielded the well defined fibrils shown in Figs. 4 and 5 when dialyzed against water. The precipitated collagen was dissolved in the same phosphate buffer used to make the original solution and again precipitated by dialysis to exhaustion of the inorganic electrolyte for a total of three cycles. Fig. 5 shows an electron micrograph of the resulting fibrils. In this case the spacing superimposed on the normal 640-Å spacing shows a longer period than previously. In the course of this repeated dissolving and precipitating the concentration of collagen in solution dropped, as previously discussed, from 0.57 to 0.08%; and it becomes questionable as to whether any mucoprotein or mucopolysaccharide present in the original solution

have survived in sufficient concentration to play a role in fibril formation unless it was firmly complexed with the collagen in solution.

To further study the effect of method of reconstitution on fibril structure as





Figs. 7 and 8. Collagen fibrils precipitated from solution in phosphate buffer (pH 8.4) at 0.33 ionic strength by salting-out with ammonium sulfate; redissolving in the same buffer and reprecipitating for a total of four times.

observed in the electron microscope, the collagen of another portion of the original solution in phosphate buffer was reconstituted by removing solvent from the system by dialyzing against Carbowax*, a high molecular-weight solid polyethylene glycol[11]. In this case only fibrils with 640-Å spacing and uniform width appeared. Also, collagen precipitated by Carbowax is completely insoluble so that repeated fractionation is not possible.

Another dissolving and precipitating sequence was carried out using ammonium sulfate as the precipitating agent. To a portion of the phosphate buffered collagen solution used in the previous sequences, saturated ammonium sulfate solution was added until the concentration of ammonium sulfate reached 35 %. This shifted the pH to the acid range, increased the ionic strength of the solution considerably and produced fibrils that gave the electron micrograph shown in Fig. 6, and while these differ considerably from the classical picture of collagen fibrils, they do possess a definite periodicity. This reconstituted collagen was again dissolved in the same phosphate buffer and again precipitated with ammonium sulfate and this cycle was carried through a total of four times. This produced the fibrils already shown in Figs. 4 and 5 plus a fibrillar form resembling a long bundle of parallel strands which show a fine striated pattern with each bundle flaring to a knot which shows incipient structure at about 3500-Å intervals[12]. Its electron micrograph (Fig. 7) shows structural features that are strikingly different from any other fibrillar form of collagen. There were also present the fibrils shown in the electron micrograph in Fig. 8 which have the regular 640-Å spacing plus partially formed flared portions.

The second phase of this study considered the results of reconstituting collagen dissolved in citrate buffer in the acid pH range. Fig. 9 is an electron micrograph of collagen dissolved in citrate buffer (pH 3.8) at 2.40 ionic strength and reconstituted by dialysis to exhaustion of the inorganic electrolyte. The fibers are much thicker than the ones obtained from a phosphate buffer solution. In Fig. 10 is shown an electron micrograph of collagen dissolved in citrate buffer that was reconstituted by adding ammonium sulfate until the concentration of this salt reached 35 %. The tangled faintly striated fibrils that result when collagen dissolved in citrate buffer is salted-out with ammonium sulfate differ considerably from the wide, flat plaque structure that results when collagen dissolved in phosphate buffer has been treated the same way as shown in Fig. 6.

Additional differences between collagen dissolved in citrate buffer at an acid pH and collagen dissolved in phosphate buffer at an alkaline pH can be demonstrated. When a solution of collagen in alkaline phosphate buffer was dialyzed against a solution of citrate buffer in the acid range a curd-like precipitate formed but it had no striated fibrils. When HIGHBERGER, GROSS AND SCHMITT carried out this procedure they obtained a collagen precipitate composed of broad flat segments and called this fibrillar form segment long-spacing[13].

When the reverse process was carried out and a solution of collagen in acid citrate buffer was dialyzed against an alkaline phosphate buffer, a precipitate of striated fibrils of small diameter was produced. When the slurry that resulted from this procedure was dialyzed to exhaustion of the inorganic electrolyte uniform fibres with the classical 640-Å spacing were produced.

* Mention of commercial products does not constitute an endorsement by the U.S. Department of Agriculture over others of a similar nature not mentioned.

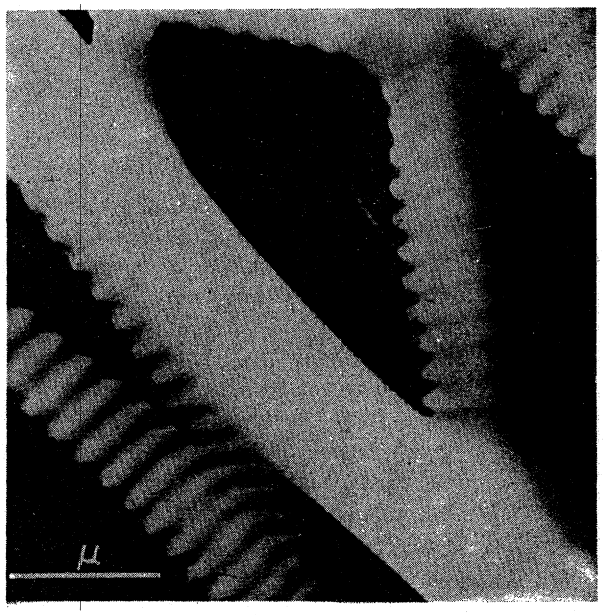


Fig. 9. Collagen fibril precipitated from solution in citrate buffer (pH 3.8) at 2.4 ionic strength by dialysis to exhaustion of the inorganic electrolyte.

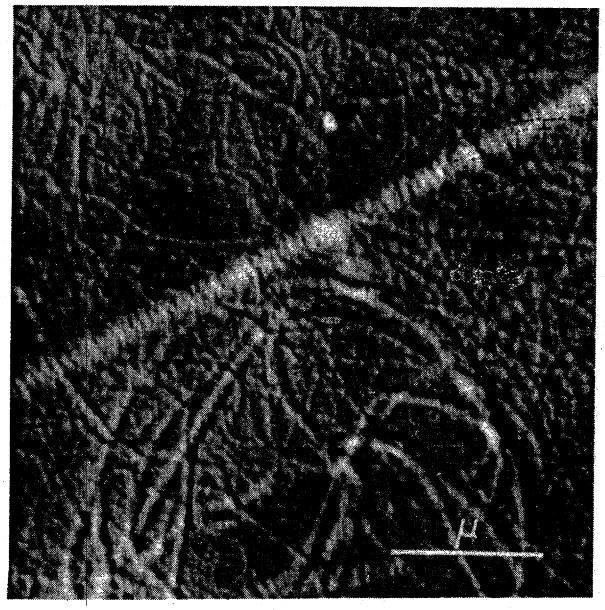


Fig. 10. Collagen fibrils precipitated from solution in citrate buffer (pH 3.8) at 2.4 ionic strength by salting-out with ammonium sulfate.

When a solution of collagen in alkaline phosphate buffer was dialyzed against 5 % sodium chloride solution no precipitate formed. However, when a solution of collagen in citrate buffer at an acid pH was dialyzed against 5 % sodium chloride solution, striated fibrils of various diameters appeared.

To minimize any effects due to the presence of mucoproteins and mucopolysaccharides, and thus emphasize that differences in fibril structure are brought about by the buffer ions present, one collagen solution was prepared in which removal of high molecular-weight compounds of these types was attempted by preliminary extraction of the hide with disodium phosphate solution and sodium chloride solution. Over a period of 7 days 1700 g of hide was extracted with eight portions of 0.2 *M* disodium phosphate of approx. 2 l each. This was followed by extraction with two portions of 1 % sodium chloride of approx. 2 l each over a period of 24 h. The hide was then washed in repeated changes of water until the washings gave negative tests for phosphate and chloride ions. The collagen in the hide was solubilized in citrate buffer (pH 3.8) at 2.40 ionic strength, and then reconstituted by dialysis to exhaustion of the electrolyte. When viewed in the electron microscope the reconstituted material consisted of very fine fibrils having only 640-Å spacing. This reconstituted collagen was then dissolved in phosphate buffer (pH 7.0) at 0.33 ionic strength and again subjected to dialysis to exhaustion of the electrolyte. Now the reconstituted collagen showed both 640-Å and 3000-Å spacings and closely resembled the material shown in Fig. 4.

DISCUSSION

What has been shown in the figures indicates that a great variety of fibrillar forms can be achieved by reconstituting collagen from solutions of different electrolytic composition. The inorganic ions present in a solution of collagen undoubtedly have many effects. These can be classified as ion binding which alters the surface-charge pattern of the dissolved protein, or as shielding effects which modify the electrostatic interaction between collagen molecules and between neighboring ionizable groups on the same collagen molecule. By preparing solutions of ichthyocolcollagen in phosphate buffer labeled with ^{32}P, GLIMCHER AND KRANE[14] have shown via measurements of radioactivity that phosphate ions are bound very firmly to collagen in solution. The combined electrostatic effects influence the aggregation of collagen molecules during the reconstitution step so that a given system consisting of collagen and inorganic electrolyte in water gives characteristic fibrils upon reconstitution.

The solubilization of collagen that occurs when electrolytic salts are used as dispersing agents can be explained in several ways. A structure that is stabilized by intermolecular hydrogen bonds can be disrupted by the presence of unbound ions which compete with the electrical charges forming the hydrogen bonds. Furthermore, the inorganic salts used as dispersing agents for collagen (phosphates, citrates, etc.) have very large activity coefficients in aqueous solution. In accordance with the Gibbs–Duhem equation, addition of these electrolytes to an aqueous solution reduces the activity of the water present[15] and in this way augments the electrostatic effect. Intramolecular hydrogen bonds are usually not broken under these conditions because being folded within the molecule they are not readily accessible, and once an intermolecular hydrogen bond is disrupted the electrical charges are free to form an intramolecular hydrogen bond that remains stable. Therefore, the over-all effect of the

inorganic electrolyte is to create intramolecular hydrogen bonds at the expense of intermolecular hydrogen bonds. This is made evident by the fact that electrolyte salts protect proteins in solution against denaturation.

Another effect of inorganic ions that relates to proteins in solution is electrostatic interaction due to fluctuating charge[16]. In the case of most proteins, and this is especially true of collagen, the number of basic residues exceeds the number of protons bound and there are many exposed ionized carboxyl groups. This gives rise to a rapid exchange of protons and a resulting time-average multipole.

Having brought about the solution of collagen from its native state by breaking intermolecular bonds so that large fibers go into solution as collagen molecules which retain a definite structure, the next step to be considered is reconstitution of the dissolved collagen by dialysis to exhaustion of the inorganic electrolyte. Reconstitution in this way seems to be a reversible process provided the dissolving and redissolving is carried out at a low concentration of electrolyte. At high concentrations of electrolyte there seems to be a permanent cross-linking which results in material that does not redissolve. A possible explanation is that the reconstituted fibril is held together by bonds which are not exclusively hydrogen bonds. Some of them may be salt bridges involving ions supplied by the buffer electrolyte which bind to the collagen in solution.

The portion of the reconstituted collagen that does not redissolve cannot be attributed to denaturation because in the electron microscope it shows the same structure and striation pattern as the collagen that redissolves. If this were a case of denaturation the secondary structure of the collagen molecule would be disrupted, and since tertiary and quaternary structure of the collagen molecule depend on secondary structure there would result, in the case of denaturation, no regularity of structure, or at least a very different structure than is found in the case of the soluble collagen.

The binding of inorganic ions to collagen molecules in solution can also explain the variety of fibrils that result from reconstitution experiments and which are shown in the figures. Bound ions can block off sites that would normally lead to one type of cross-linking, and ion binding could also create new sites which lead to their own characteristic types of cross-linking. Also, the relative numbers of salt linkages and hydrogen bonds would be influenced by the nature and concentration of the free charges present in the solution.

The progressive insolubility of collagen that results when it is repeatedly dissolved and reconstituted, plus the failure of dialysis to exhaustion of the electrolyte to completely erase the effects of previous contact with inorganic salts, indicate that removal of ions bound to collagen is difficult. If bound ions were easily removed, all collagen solutions which had been dialyzed to exhaustion of the electrolyte would show the same structure regardless of previous electrolytic environment.

Some exchange of ions, however, probably does take place and accounts for the difference in fibrillar structure that results when a solution of collagen in phosphate buffer is reconstituted by dialysis to exhaustion of the electrolyte as shown in Fig. 4, and when it is reconstituted by salting-out with ammonium sulfate as shown in Fig. 6. A possible explanation of this difference is that ions from the added ammonium sulfate replaced phosphate ions already bound to the collagen and in turn gave rise to new types of cross-linkage. This reasoning may be extended to the forms in Figs. 7 and 8 which come from the same solution after it has gone through the dissolving and

reconstituting cycle three more times; and also to Fig. 9 where are shown fibrils reconstituted from a solution of collagen in citrate buffer by dialysis to exhaustion of the electrolyte, and in Fig. 10 fibrils reconstituted from the same solution by salting-out with ammonium sulfate.

The material presented here has been an attempt to explain the solution and precipitation behavior of collagen on the basis of interaction between inorganic ions and collagen molecules in solution. There is still much to be explained, but it appears that the configuration of the collagen molecule in solution and the type of fibril that is reconstituted depend on previous solution history.

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